Inflammasome-independent Role of Apoptosis-associated Speck-like Protein Containing a CARD (ASC) in T Cell Priming Is Critical for Collagen-induced Arthritis*[S]

Sirish K. Ippagunta[‡1], **David D. Brand**[§1], **Jiwen Luo**[§], **Kelli L. Boyd**[‡], **Christopher Calabrese**[‡], **Rinke Stienstra**[¶], **Frank L. Van de Veerdonk**[¶], **Mihai G. Netea**[¶], **Leo A. B. Joosten**[¶1], **Mohamed Lamkanfi**[||1,2], and **Thirumala-Devi Kanneganti**[‡1,3]

From the ‡Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, §Research Service, Veterans Affairs Medical Center, University of Tennessee, Memphis, Tennessee 38104, the ¶Department of Medicine, Radboud University Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands, and the ||Department of Biochemistry, Department of Medical Protein Research, Ghent University, VIB, B-9000 Ghent, Belgium

Rheumatoid arthritis is an autoimmune disease with 1% prevalence in the industrialized world. The contributions of the inflammasome components Nlrp3, ASC, and caspase-1 in the pathogenesis of collagen-induced arthritis have not been characterized. Here, we show that ASC$^{-/-}$ mice were protected from arthritis, whereas Nlrp3$^{-/-}$ and caspase-1$^{-/-}$ mice were susceptible to collagen-induced arthritis. Unlike Nlrp3$^{-/-}$ and caspase-1$^{-/-}$ mice, the production of collagen-specific antibodies was abolished in ASC$^{-/-}$ mice. This was due to a significantly reduced antigen-specific activation of lymphocytes by ASC$^{-/-}$ dendritic cells. Antigen-induced proliferation of purified ASC$^{-/-}$ T cells was restored upon incubation with wild type dendritic cells, but not when cultured with ASC$^{-/-}$ dendritic cells. Moreover, direct T cell receptor ligation with CD3 and CD28 antibodies induced a potent proliferation of ASC$^{-/-}$ T cells, indicating that ASC is specifically required in dendritic cells for antigen-induced T cell activation. Therefore, ASC fulfills a hitherto unrecognized inflammasome-independent role in dendritic cells that is crucial for T cell priming and the induction of antigen-specific cellular and humoral immunity and the onset of collagen-induced arthritis.

Rheumatoid arthritis is an autoimmune disease with 1% prevalence in the industrialized world characterized by chronic inflammation of the synovial joints and a progressive destruction of articular tissue. Immunization of mice with complete Freund's adjuvant and native type II collagen (CII),[4] a major component of articular cartilage, triggers arthritis with clinical and pathological features similar to rheumatoid arthritis in humans (1–3). Similarities between mouse CIA and human rheumatoid arthritis include the linkage of disease to genes located in the histocompatibility locus, mononuclear cell infiltration, pannus development, fibrin deposition, cartilage erosion, and bone destruction. The induction of both humoral and T cell immunity against CII are induced during collagen-induced arthritis (CIA) and have been demonstrated to be essential for the onset and severity of the disease (4).

The discovery of NOD-like receptors (NLRs) as essential components of the immune system triggered significant interest in the study of their contribution to the pathogenesis of autoimmune diseases. NLRs comprise a large family of intracellular proteins that are believed to be primarily involved in the innate immune response to microbial pathogens through the recognition of conserved pathogen-associated molecular patterns (5–8). However, they also contribute by sensing "danger signals," *i.e.* endogenous molecules that are produced during tissue damage or inflammation (8–11).

A prominent example of an NLR protein implicated in autoinflammatory disease is Nlrp3 (also called Nalp3/cryopyrin; 12). Nlrp3 allows for the recruitment and autocatalytic activation of the cysteine protease caspase-1 (Casp1) in a large cytosolic protein complex named the "inflammasome" (7, 13). The bipartite adaptor protein ASC bridges the interaction between Nlrp3 and caspase-1 by means of homotypic interactions involving its pyrin and CARD motifs, making it essential for activation of the inflammasome. However, the role of the inflammasome components Nlrp3, ASC, and caspase-1 in CIA has not been addressed. The goal of the present study was to understand the roles of Nlrp3, ASC, and caspase-1 in CIA. We found that ASC knock-out mice were protected from arthritis, whereas disease incidence and severity in Nlrp3$^{-/-}$ and caspase-1$^{-/-}$ mice was normal. Unlike Nlrp3$^{-/-}$ and caspase-1$^{-/-}$ mice, the production of CII-specific antibodies in the serum of immunized ASC$^{-/-}$ mice was abolished. In addition, antigen-specific proliferation and production of T cell cytokines by ASC$^{-/-}$ lymph node cells was significantly reduced, whereas antigen-independent T cell activation was unaffected. Incubation of ASC$^{-/-}$ T cells with wild type, but not ASC$^{-/-}$

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[1] These authors contributed equally to this work.

[3] To whom correspondence should be addressed: Dept. of Immunology, St. Jude Children's Research Hospital, MS #351, 570, St. Jude Place, Suite E7004, Memphis TN 38105-2794. Tel.: 901-495-3634; Fax: 901-495-5766; E-mail: Thirumala-Devi.Kanneganti@StJude.org.

[4] The abbreviations used are: CII, chicken type II collagen; CIA, collagen-induced arthritis; WT, wild type; Casp1, caspase-1; DC, dendritic cell; IL, interleukin; NLR, NOD-like receptor; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; IFN, interferon; micro-CT, microcomputed tomography; CFA, complete Freund's adjuvant.
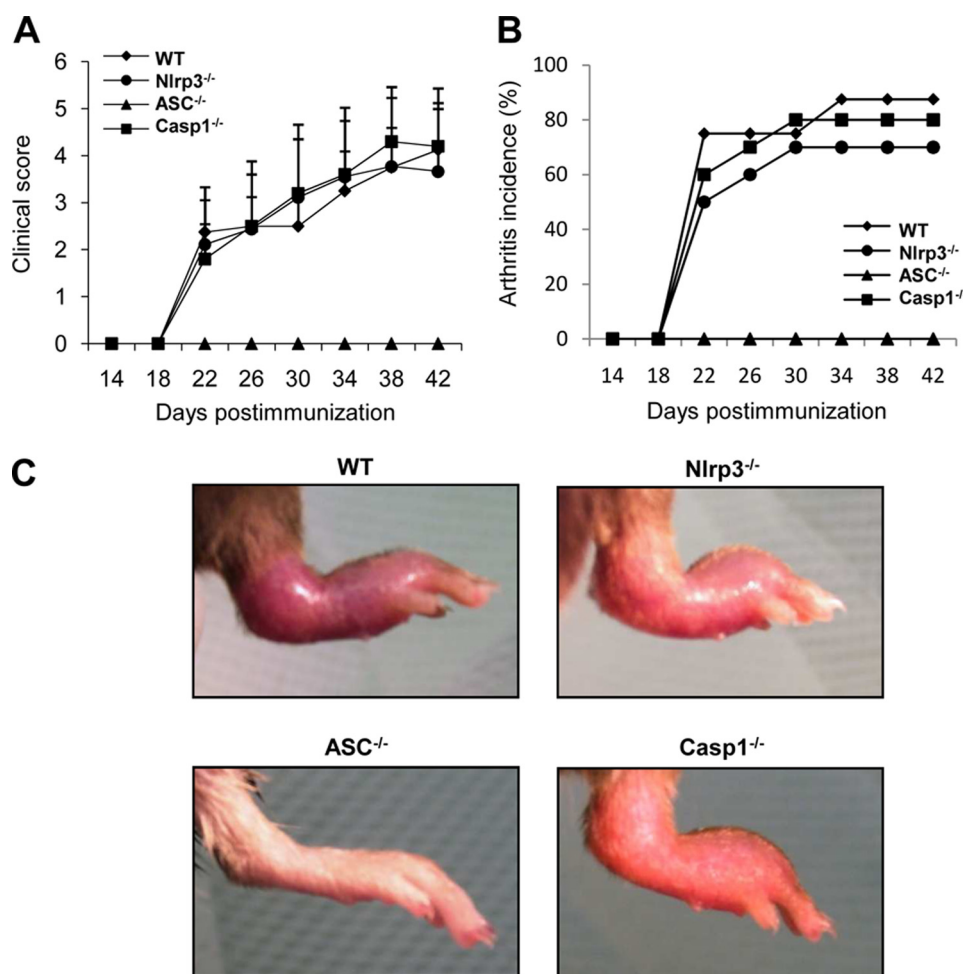
FIGURE 1. **Clinical arthritis symptoms in CII-immunized Nlrp3$^{-/-}$, ASC$^{-/-}$, and caspase-1$^{-/-}$ mice.** *A*, mean clinical scores + S.E. of arthritis in C57BL/6 wild type ($n = 8$), Nlrp3$^{-/-}$ ($n = 10$), ASC$^{-/-}$ ($n = 10$), and Casp1$^{-/-}$ ($n = 10$) mice immunized with CII emulsified in CFA. *B*, percentage of animals of each genotype that developed arthritis in at least 1 digit. *C*, hind paws of CII-immunized WT, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice. Images are representative of at least six mice in each group. The results are representative for three separate experiments.

dendritic cells (DCs), restored antigen-induced proliferation, hence confirming that ASC expression in dendritic cells is required for T cell priming. Therefore, ASC fulfills a caspase-1- (and thus inflammasome)-independent role in T cell priming in dendritic cells that is crucial for induction of antigen-specific cellular and humoral immunity and the onset of collagen-induced arthritis.

EXPERIMENTAL PROCEDURES

Mice—Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice backcrossed to C57BL/6 background for at least 10 generations have been described before (14, 15). Mice were housed in a pathogen-free facility, and the animal studies were conducted under protocols approved by the St. Jude Children's Research Hospital Committee on Use and Care of Animals.

Induction and Assessment of Arthritis—CII was prepared according to the methods of Miller *et al.* (16) dissolved in 10 mM acetic acid (4 mg/ml overnight at 4 °C) and emulsified in an equal volume of CFA containing 4 mg/ml heat-killed *Mycobacterium tuberculosis* (H37 Ra, Difco). Arthritis was induced as described previously (17) with a single intradermal injection of

200 mg CII/100 ml emulsion. The severity of the arthritis was assessed using a visual scoring system. Each paw was scored on a graded scale from 0 to 3: 0, normal paw; 1, swelling and/or redness of one toe or finger joint; 2, swelling of two or more toes or joints, or increased swelling; and 3, severe swelling and/or ankylosis throughout the entire paw. Each paw was graded, and the four scores were added such that the maximal score per mouse was 12. The clinical score was determined daily for each mouse for up to 42 days.

Histology—Hind limbs were fixed for 24 h in 10% neutral buffered formalin and decalcified in TBD-2. Knee joints were processed by routine methods to paraffin blocks. Specimens were sectioned at 6 mm and stained with hematoxylin and eosin. Safranin-O staining was performed to evaluate cartilage integrity. The stifle joint sections were evaluated for disease severity using a scoring system from 0 to 4: 0, within normal limits; 1, minimal; 2, mild; 3, moderate; and 4, severe. Evaluation and scoring was performed by an experienced veterinary pathologist.

Micro-CT Imaging—Micro-CT micrographs of paws fixed in formalin were made using an *ex vivo* micro-CT scanner (LocusSP Specimen CT, GE Healthcare) at 28-μm isotropic voxel size, with 720 projections, an integration time of 1,700 msec, photon energy of 80 keV, and a current of 70 μA.

Anti-type II Collagen Antibody Enzyme-linked Immunosorbent Assay—IgG antibody levels against the immunogen were measured by standard ELISA methodology. In brief, 96-well serocluster U-vinyl plates (Costar) were coated with 100 μl of CII (5 μg/ml) overnight at 4 °C. The wells were blocked with 200 μl of PBS/Tween 20 containing 2% (w/v) bovine serum albumin (1 h at 4 °C). 2-Fold serial dilutions (beginning at 1/100 dilution) of mouse sera in PBS supplemented with 0.1% (v/v) Tween 20 and 2% normal goat serum (PelFreeze) were applied and incubated for 2 h at 37 °C. Horseradish peroxidase-conjugated goat anti-mouse isotype-specific IgG1, IgG2b, IgG2c, IgG3, or IgM antisera (Southern Biotechnology) were applied for 2 h at 4 °C, and conversion of the substrate (*o*-phenylenediamine dihydrochloride, Sigma) was determined using a microplate reader (Bio-Rad model 450).

T Cell Proliferation Assays—Popliteal lymph nodes were collected 10 days after immunization with 20 μl of CFA emulsion containing 10 μg of either CII or ovalbumin (Sigma). CD4$^+$ T
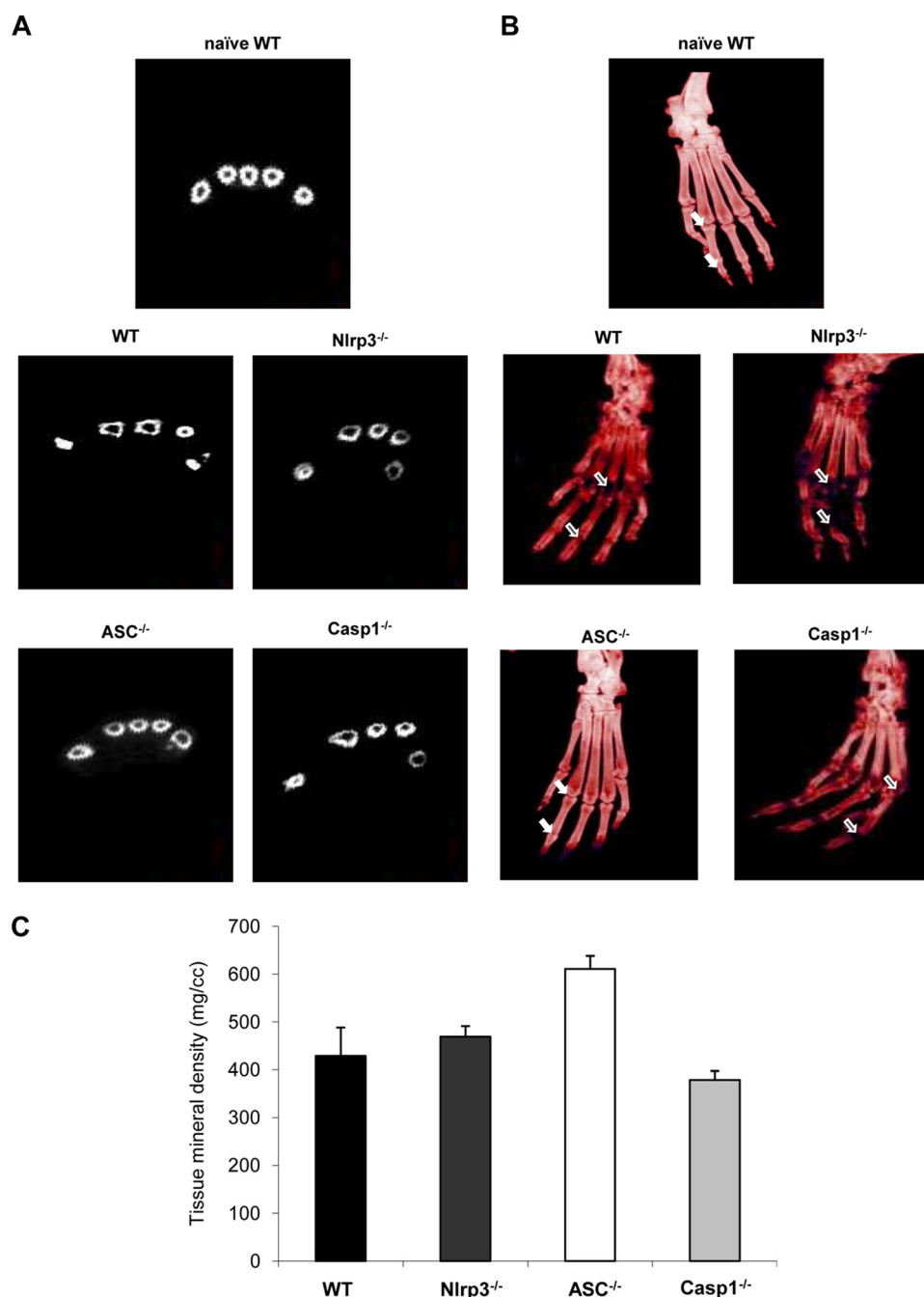
FIGURE 2. **Analysis of bone destruction using micro-computed tomography.** *A*, axial micro-computed tomography (micro-CT) image cross-sections of mouse hind limbs of CII-immunized WT, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice showing the second, third, and fourth metatarsals and first and fifth proximal phalanges. All images are representative of at least six mice in each group. *B*, three-dimensional micro-CT surface renderings of hind paws of CII-immunized WT, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice. The *solid white arrow* indicates intact cartilage and bone, and the *gray arrow* indicates bone and cartilage erosion. All images are representative of at least six mice in each group. *C*, tissue mineral density of the hind paw digits of CII-immunized WT, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice. Data represent mean $\pm$ S.E. of all immunized mice of each genotype ($n = 6$).

the indicated concentration of antigen. Cultures were maintained in U-bottom plates in 300 μl of HL-1 medium (Lonza) supplemented with 50 units/ml penicillin G and 50 μg/ml streptomycin (Invitrogen), 50 μM 2-mercaptoethanol, 292 μg/ml L-glutamine, and 0.1% bovine serum albumin, at 37 °C, 5% CO$_2$ for 72 h. Supernatants were harvested for evaluation of cytokine production (below), and cultures were pulsed with [^{3}H]thymidine (1 μCi/well in 10 μl), incubated for an additional 18 h, harvested onto Unifilter GF/C plates, and counted (counts/min on a TopCount NXT (PerkinElmer).

Generation and Analysis of Bone Marrow-derived Dendritic Cells— Bone marrow-derived dendritic cell cultures were prepared as described (18) and stimulated with lipopolysaccharide (*Escherichia coli*, serotype 0111:B4, Sigma) or heat-killed *Mycobacterium* (1 μg/ml) or PBS for 24 h. Bone marrow-derived dendritic cell cultures were resuspended into PBS/2% fetal bovine serum, stained with fluorescein isothiocyanate-conjugated anti-CD11c monoclonal antibody, PE-conjugated anti-MHC class II monoclonal antibody and APC-conjugated anti-CD86 on ice for 30 min, washed twice with PBS/2% fetal bovine serum, and fixed in PBS/2% paraformaldehyde. Labeled cells were assayed with a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo software.

Cytokine Quantification—IL-2, IL-10, IL-13, IL-17, and IFN-γ were determined using a mouse Bio-Plex ELISA kit (Bio-Rad).

Statistical Analysis—Data are represented as the mean $\pm$ S.E. Clinical and histopathological scores and serum anti-CII antibody levels were analyzed with Student's *t* test. Incidence of mice that developed disease

cells were isolated by negative selection using a mouse CD4$^+$ T cell enrichment strategy (Miltenyi AutoMACS). CD4$^+$/CD11c$^+$ dendritic cells were isolated from collagenase-D-treated (Roche) spleens of naïve ASC$^{-/-}$ and ASC$^{+/+}$ mice according to the manufacturer's instructions. Cultures consisted of with 5×10^5 of the indicated type of CD4 T cells/well and 2.5×10^5 of the indicated type of dendritic cells/well and

was analyzed with Fisher's exact test. *p* values < 0.05 were considered significant.

RESULTS

ASC Is Required for Collagen-induced Arthritis Independently of Its Role in Caspase-1 Activation—To determine the functional roles of Nlrp3, ASC, and Casp1 in the development
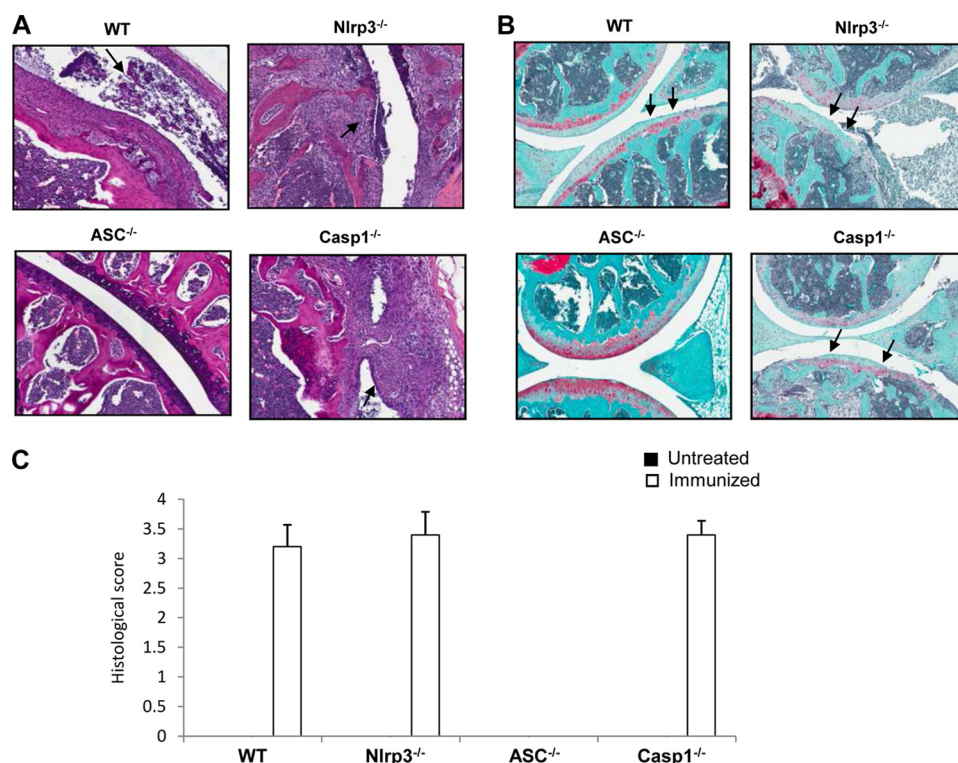
FIGURE 3. **Protection from histopathological signs of arthritis in ASC$^{-/-}$, but not in Nlrp3$^{-/-}$ or Casp1$^{-/-}$ mice.** *A* and *B*, micrographs of hematoxylin and eosin- (*A*) and safranin-O- (*B*) stained sections of stifle joints of CII-immunized WT, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice. *Arrows* indicate cell infiltrations (*A*) and dead chondrocytes and cartilage erosion (*B*). *C*, histological grading of synovial hypertrophy and degradation of cartilage and bone in all CII-immunized WT, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice. Data represent mean ± S.E. of all immunized mice of each genotype. All images are representative of at least six mice in each group.

and progression of CIA, arthritic symptoms in age- and sex-matched wild type and gene-deficient mice lacking Nlrp3, ASC, or caspase-1 were studied following immunization with CII on day 0. Mice were examined daily for signs of developing arthritis. The severity of arthritis was assessed using a visual scoring system. The clinical scores and the arthritis incidence in Nlrp3$^{-/-}$ and caspase-1$^{-/-}$ were comparable with those of wild type mice (Fig. 1, *A* and *B*). By day 42, 70–80% of the immunized wild type, Nlrp3$^{-/-}$, and caspase-1$^{-/-}$ mice had a clinical score >3. Remarkably, all mice in the ASC knock-out cohort were resistant to CIA, with none of the mice in this cohort displaying any clinical signs of arthritis at day 42 (Fig. 1, *A* and *B*). Indeed, the hind paws of most wild type, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ mice were significantly swollen and presented with ankylosis at day 34, whereas the hind paws of all immunized ASC knock-out mice had a normal appearance (Fig. 1*C*).

To validate these clinical assessments, the extent of bone and cartilage erosion in hind paws of representative wild type, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice was assessed by three-dimensional micro-CT imaging. Hind paws of immunized WT, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ mice exhibited severe cortical bone erosion in the metatarsal region (Fig. 2, *A* and *B*). In contrast, hind paws of CII-immunized ASC$^{-/-}$ mice appeared similar to those of naïve mice (Fig. 2, *A* and *B*) and displayed no apparent bone erosion (Fig. 2, *A* and *B*). To compare the extent of bone destruction, we quantified the mineral density of the five hind

paw digits of CII-immunized mice of each genotype. In line with the micro-CT images, digits of immunized ASC knock-out mice were significantly more dense than those of wild type, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ mice (Fig. 2*C*).

The micro-CT studies were subsequently complemented with histological examination of representative stifle joints. Unlike ASC$^{-/-}$ mice, hematoxylin and eosin-stained tissue sections of the stifle joints of wild type, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ mice showed substantial levels of infiltrated inflammatory cells, thickening of the lining layer and chondrocyte cell death (Fig. 3*A*). Significant cartilage and subchondral bone erosion and depletion of matrix proteoglycan was apparent on safranin O-stained tissue sections of each genotype, except ASC$^{-/-}$ mice as evidenced by the deep pink color of the cartilage (Fig. 3*B*). Semiquantitative scoring of these histological parameters confirmed that arthritic severity in Nlrp3$^{-/-}$ and Casp1$^{-/-}$ was comparable with those of wild type mice, whereas ASC$^{-/-}$ mice were strongly protected from CIA histopathology (Fig. 3*C*). Together, these results demonstrate that ASC is required for the development of CIA independently of its role in caspase-1 activation in the inflammasome.

The Induction of Humoral Immunity during Collagen-induced Arthritis Requires ASC, but Not Nlrp3 or Caspase-1—Both T cell-mediated and humoral immune responses have been demonstrated to play an essential role in the pathogenesis of CIA (3–4, 20). To gain some insight into the mechanism by which ASC mediates CIA, we first determined the serum levels of CII-specific antibodies in immunized mice at day 42 post-immunization. Circulating levels of CII-specific IgG1, IgG2b, IgG2c, and IgG3 antibodies were comparably elevated in the serum of wild type, Nlrp3$^{-/-}$ and Casp1$^{-/-}$ mice (Fig. 4, *A*–*D*). Moreover, the serum of immunized ASC$^{-/-}$ mice was devoid of CII-specific IgM antibodies (Fig. 4*E*), indicating that ASC functions upstream of antibody class switch recombination. In agreement with these findings, the majority of WT, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ mice presented with significantly enlarged popliteal lymph nodes, whereas none of the immunized ASC$^{-/-}$ mice had clearly visible popliteal lymph nodes (Fig. 5*A*). Concurrently, popliteal lymph nodes of ASC knock-out mice were nearly devoid of immune cells, whereas those of immunized wild type, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ mice contained several million cells per lymph node (Fig. 5*B*). These results demonstrate that unlike wild
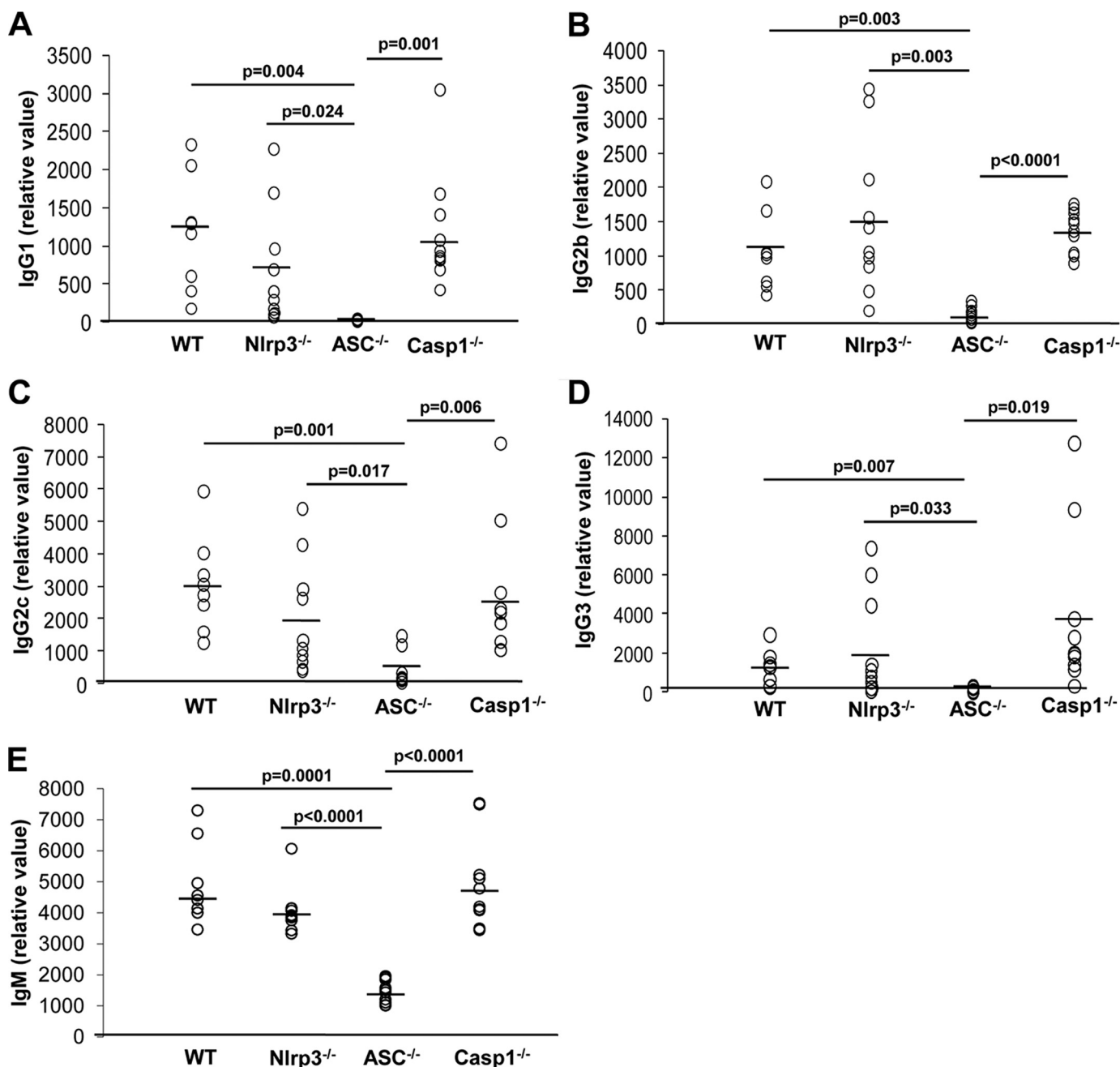
FIGURE 4. **Defective humoral immune response in CII-immunized ASC knock-out mice.** *A–D*, circulating levels of CII-specific IgG1 (*A*), IgG2b (*B*), IgG2c (*C*), IgG3 (*D*), and IgM (*E*) antibodies were determined in sera from WT ($n = 8$), Nlrp3$^{-/-}$ ($n = 10$), Casp1$^{-/-}$ ($n = 10$), and ASC$^{-/-}$ ($n = 10$) mice 42 days after immunization with CII. Data represent mean ± S.E. of all immunized mice of each genotype.

type, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ mice, animals lacking ASC are incapable of mounting a humoral immune response to CII.

ASC Is Required for the Induction of Antigen-specific Lymphocyte Proliferation—Induction of a CII-specific humoral immune response relies on activation of T cells by antigen-presenting cells in the presence of the immunizing antigen. We therefore investigated whether the lack of anti-CII antibodies in immunized ASC$^{-/-}$ mice was due to a defective T cell response to CII. *In vitro* proliferation of lymph node cells from ASC$^{-/-}$ mice in the presence of CII was significantly impaired relative to those of ASC$^{+/+}$ mice (Fig. 6*A*). The

defective antigen-specific proliferation of ASC$^{-/-}$ T cells is not limited to CII as the immunizing antigen because ASC$^{-/-}$ lymph node cells also failed to proliferate in the presence of tuberculin-purified protein derivative (Fig. 6*B*) and ovalbumin (Fig. 6*C*). In agreement with the defective antigen-specific proliferation of lymph node cells from ASC$^{-/-}$ mice immunized with ovalbumin in CFA (Fig. 6*C*), the supernatant of ovalbumin-restimulated ASC$^{-/-}$ lymph node cells contained significantly reduced levels of the T_H1 cytokines IFN-γ and IL-2 (Fig. 6, *D* and *E*), the T_H2 cytokines IL-10 and IL-13 (Fig. 6, *F* and *G*) and the T_H17 cytokine IL-17 (Fig. 6*H*). Unlike ASC-deficient lymphocytes, lymph node
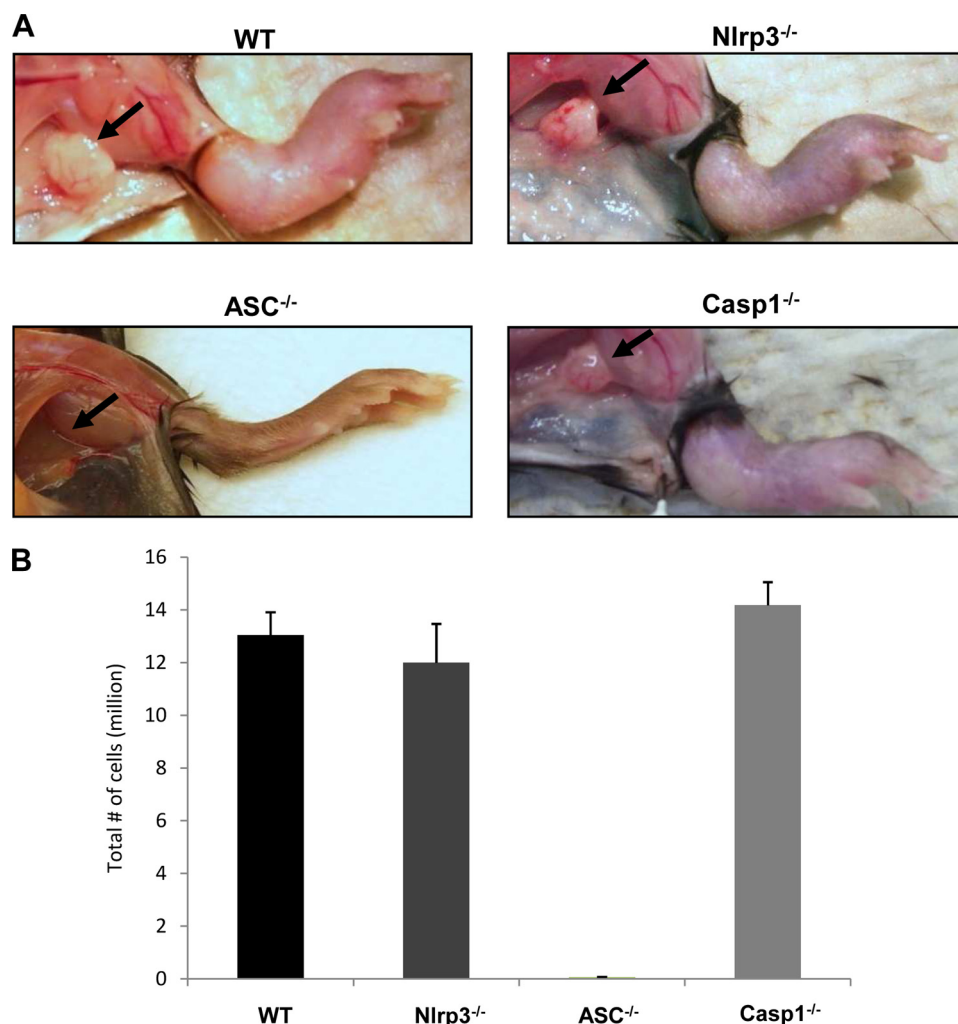
A

B

FIGURE 5. **ASC knock-out mice are protected from CII-induced popliteal lymph node enlargement.** *A*, images of representative popliteal lymph nodes in CII-immunized WT, Nlrp3$^{-/-}$, Casp1$^{-/-}$, and ASC$^{-/-}$ mice. All images are representative of at least six mice in each group. *B*, total cell count in a single popliteal lymph node of CII-immunized WT, Nlrp3$^{-/-}$, ASC$^{-/-}$, and Casp1$^{-/-}$ mice. Data represent mean ± S.E. of all immunized mice of each genotype ($n = 4$).

ASC Expression in Dendritic Cells Is Required for T Cell Priming—To test the hypothesis that antigen-specific lymphocyte proliferation requires ASC expression in dendritic cells, ovalbumin-induced proliferation of purified ASC$^{+/+}$ or ASC$^{-/-}$ T cells was measured following incubation with isolated ASC$^{+/+}$ or ASC$^{-/-}$ dendritic cells. Our results show that wild type T cells incubated with wild type dendritic cells mounted a significant proliferative response to ovalbumin (Fig. 7*A*). ASC$^{-/-}$ T cells co-cultured with wild type dendritic cells displayed a proliferative response comparable with that of wild type T cells incubated with wild type dendritic cells (Fig. 7*A*), demonstrating that ASC$^{-/-}$ T cells can mount a normal ovalbumin-induced proliferative response in the presence of wild type dendritic cells. In contrast, both wild type and ASC$^{-/-}$ T cells co-cultured with ASC$^{-/-}$ dendritic cells exhibited a substantially reduced ovalbumin-induced proliferation (Fig. 7*A*). We also analyzed the expression of dendritic cell activation markers to ensure that ASC deficiency did not cause a general defect in dendritic cell responsiveness. In line with a specific role for ASC in T cell priming, cell surface expression of MHCII and CD86 were similarly induced in LPS-treated wild type and ASC-deficient dendritic cells relative to PBS-treated controls (supplemental Fig. 4). Cell surface expression of MHCII and CD86 was also comparably induced in ASC$^{-/-}$ and wild type dendritic cells stimulated with heat-killed *M. tuberculosis* (supplemental Fig. 5), an immunostimulating component of CFA. In contrast to ASC$^{-/-}$ dendritic cells, the absence of the inflammasome components Nlrp3 (Fig. 7*B*) and Casp1 (Fig. 7*C*) in dendritic cells did not affect ovalbumin-induced T cell proliferation. These results demonstrate that ASC expression in dendritic cells is required for antigen-induced T cell priming independently of caspase-1 inflammasomes. Moreover, the impaired antigen presentation by ASC$^{-/-}$ dendritic cells, but not Nlrp3$^{-/-}$ or Casp1$^{-/-}$ dendritic cells, correlates strongly with the defects in antigen-induced adaptive immunity and the marked resistance against collagen-induced arthritis observed in ASC$^{-/-}$ mice, but not Nlrp3$^{-/-}$ or Casp1$^{-/-}$ mice. These data demonstrate that ASC fulfills a caspase-1 inflammasome-independent role in T cell priming in dendritic cells that is crucial for induction of antigen-
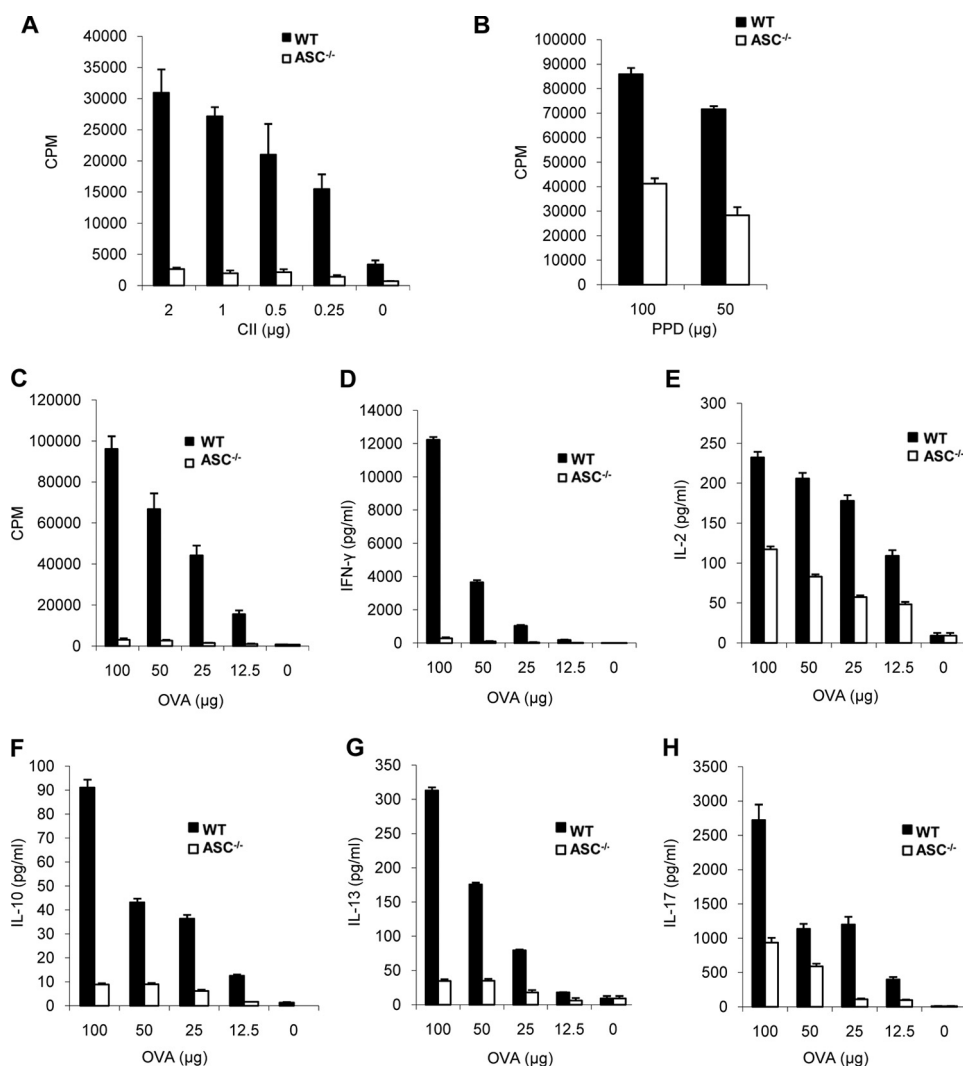
cells from Nlrp3$^{-/-}$ and Casp1$^{-/-}$ mice that were immunized with ovalbumin in CFA mounted a potent dose-dependent proliferation in response to ovalbumin (supplemental Fig. 1). The role of ASC in antigen-induced humoral and T cell responses is not strictly associated with the T$_H$1 adjuvant CFA because ASC knock-out mice immunized with ovalbumin in the T$_H$2 adjuvant alum (adjuvant containing hydrated aluminum potassium sulfate) also failed to produce ovalbumin-specific antibodies and the T$_H$2-associated cytokine IL-5 (supplemental Fig. 2). Moreover, the inhibition of proliferation in ASC$^{-/-}$ immune cells is antigen-specific because direct T cell receptor ligation with CD3 and CD28 antibodies induced a potent proliferation of ASC$^{-/-}$ CD4$^+$ and CD8$^+$ (supplemental Fig. 3) T cells. These results show that ASC-deficient T cells appear intrinsically normal and suggest that ASC fulfills an inflammatory role in antigen presentation in dendritic cells. Moreover, similar to the inflammasome-independent role of ASC in the induction of CIA, the ASC-dependent induction of antigen-specific lymphocyte proliferation does not require Nlrp3 or Casp1.

FIGURE 6. **Defective antigen-specific proliferation and cytokine production in ASC$^{-/-}$ mice.** *A–C,* dose-dependent antigen-specific proliferation of lymph node cells in response to CII (*A*), tuberculin-purified protein derivative (*PPD*) (*B*), and ovalbumin (*OVA*) (*C*). *D–G,* dose-dependent antigen-induced production of interferon-γ (*IFN-γ*) (*D*), IL-2 (*E*), IL-10 (*F*), IL-13 (*G*), and IL-17 (*H*) by lymph node cells stimulated with ovalbumin for 72 h. All data represent the mean ± S.E. of triplicates and are representative of three independent experiments.

ing that the role of ASC in CIA is independent from the caspase-1 inflammasome.

Our observation that caspase-1-deficient mice are susceptible to CIA may appear as a contradiction to a previous report demonstrating that the chemical Casp1 inhibitor VX-765 provided protection against CIA (20). However, it should be noted that VX-765 is also an effective inhibitor of other inflammatory caspases. For instance, its K_i for caspase-4 (<0.6 nM) is slightly better than the K_i for Casp1 (0.8 nM) (20). This suggests that the protection against CIA observed with VX-765 may be due to inhibition of inflammatory caspases other than caspase-1. Noteworthy in this regard is the fact that caspase-1-deficient mice very recently also have been shown to be susceptible to antigen-induced arthritis, whereas ASC knockout mice were highly resistant (21). However, one may not conclude from the observation that Casp1$^{-/-}$ mice are sensitive to CIA and antigen-induced arthritis and that there is no role for IL-1β and IL-18 in arthritis. Indeed, several additional proteases including caspase-8 (22), proteinase-3 (23), and granzymes (24) have been shown to process IL-1β and IL-18 into biologically active cytokines. Moreover, several of these proteases have been implicated in CIA (25). Thus, IL-1β and IL-18 production through one (or several) of these proteases may contribute to CIA and antigen-induced arthritis.

specific cellular and humoral immunity and the onset of collagen-induced arthritis.

DISCUSSION

The murine model of CIA has been extensively used to increase our understanding of autoimmune-mediated arthritis and identify potential new therapeutic agents to treat rheumatoid arthritis in humans (19). This study is the first to demonstrate the critical role of ASC as a key player in the pathogenesis of CIA. ASC-deficient mice were dramatically protected from the incidence and severity of disease in CIA. The absence of clinical symptoms of arthritis was confirmed by the absence of bone destruction in micro-CT scans and by histopathological evaluation. In marked contrast to ASC knock-out mice, clinical and histological evaluation of type II collagen-immunized Casp1$^{-/-}$ and Nlrp3$^{-/-}$ mice showed a normal progression of disease parameters, indicat-

To study the mechanism by which ASC controls CIA induction, we started from the knowledge that the pathogenesis of CIA is dependent on both humoral and cellular immunity against CII (4). The amount of anti-collagen type II antibodies of IgG1, IgG2, IgG3, and IgM subtypes were all significantly reduced in ASC knock-out mice. Consistent with an inflammasome-independent role for ASC in CIA onset and progression, the levels of anti-CII-antibodies in the serum of Nlrp3$^{-/-}$ and Casp1$^{-/-}$ mice did not differ significantly from those of immunized wild type mice. Dendritic cells are the most potent antigen-presenting cells capable of stimulating naïve T cells for the induction of T cell and B cell responses. Indeed, we found that antigen-specific activation of ASC-deficient T cells by ASC knock-out dendritic cells was significantly hampered. In contrast, the antigen-specific proliferation of ASC-deficient T cells following incubation with wild type dendritic cells was normal. This
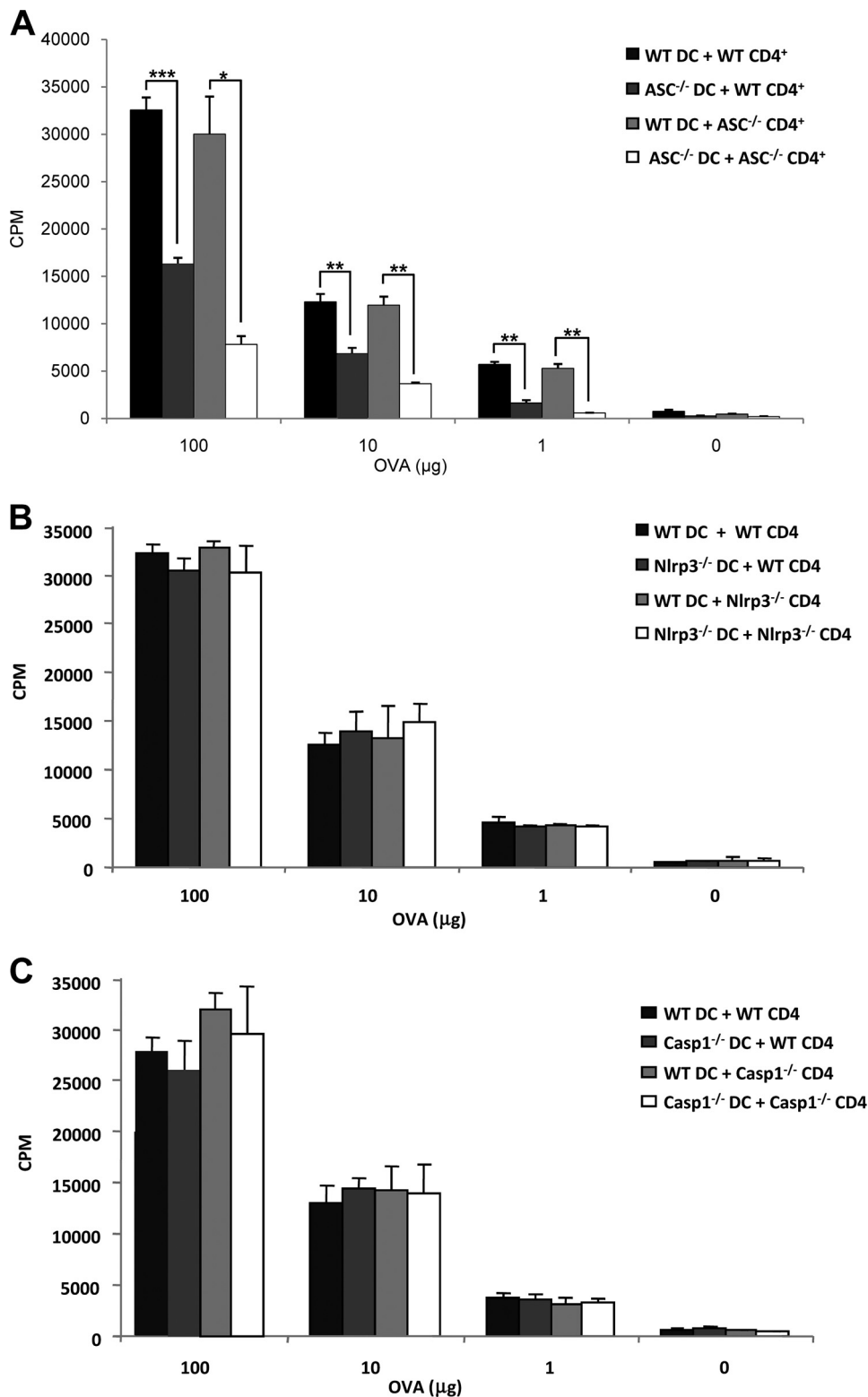
FIGURE 7. **ASC, but not Nlrp3, and caspase-1 expression in dendritic cells is essential for ovalbumin-specific lymphocyte proliferation.** *A*, dose-dependent ovalbumin (*OVA*)-induced proliferation of purified CD4$^+$ T cells from WT and ASC$^{-/-}$ mice incubated with purified WT or ASC$^{-/-}$ dendritic cells (DC). *B*, dose-dependent ovalbumin-induced proliferation of purified CD4$^+$ T cells from WT and Nlrp3$^{-/-}$ mice incubated with purified WT or Nlrp3$^{-/-}$ dendritic cells (DC). *C*, dose-dependent OVA-induced proliferation of purified CD4$^+$ T cells from WT and caspase-1$^{-/-}$ mice incubated with WT or caspase-1$^{-/-}$ dendritic cells. All data represent the mean ± S.E. of triplicates and are representative of three independent experiments. *CPM*, counts per min.

suggests that the underlying cause for the absence of CII-induced T cell and B cell responses during CIA in ASC knock-out mice was mainly due to impaired T cell priming by ASC-deficient dendritic cells. In line with this notion, direct ligation of the T cell receptor of ASC knock-out T cells with anti-CD3 and anti-CD28 antibodies triggered a potent T cell proliferation comparable with that of wild type T cells. The role of ASC in T cell priming by dendritic cells is inflammasome-independent because Nlrp3$^{-/-}$ and Casp1$^{-/-}$ dendritic cells did not display this defect in T cell priming. Moreover, the role of ASC in dendritic cells is specifically associated with T cell priming because the LPS- and heat-killed *Mycobacterium*-induced induction of the cell surface activation markers MHCII and CD86 was not affected in ASC$^{-/-}$ dendritic cells.

An inflammasome-independent role for ASC in lymphocytes during bovine serum albumin-induced arthritis was recently reported (21). Although the latter and our work are largely in agreement, we showed that ASC is required in dendritic cells for antigen-induced T cell priming. A possible explanation for this apparent discrepancy may lie in the fact that ASC may have roles in both dendritic cells and lymphocytes. Thus, depending on the experimental arthritis model under study, its role in either myeloid or lymphoid cells may prevail. On a molecular level, several mechanisms by which ASC controls antigen presentation in dendritic cells can be envisaged and require further investigation. For instance, ASC may mediate antigen processing, intracellular trafficking, and/or antigen loading onto MHC receptors in dendritic cells. Alternatively, ASC may be required for the production of co-stimulatory factors. Regardless, our results establish a novel role of ASC in the induction of adaptive immune responses independently of caspase-1 inflam-

masomes and suggest a crucial role for ASC in arthritis and other autoimmune diseases apart from the inflammasome.

REFERENCES

1. Trentham, D. E., Townes, A. S., and Kang, A. H. (1977) *J. Exp. Med.* **146,** 857–868
2. Courtenay, J. S., Dallman, M. J., Dayan, A. D., Martin, A., and Mosedale, B. (1980) *Nature* **283,** 666–668
3. Haqqi, T. M., and David, C. S. (1990) *J. Autoimmun.* **3,** 113–121
4. Holmdahl, R., Andersson, M., Goldschmidt, T. J., Gustafsson, K., Jansson, L., and Mo, J. A. (1990) *Immunol. Rev.* **118,** 193–232
5. Ting, J. P., Lovering, R. C., Alnemri, E. S., Bertin, J., Boss, J. M., Davis, B. K., Flavell, R. A., Girardin, S. E., Godzik, A., Harton, J. A., Hoffman, H. M., Hugot, J. P., Inohara, N., Mackenzie, A., Maltais, L. J., Nunez, G., Ogura, Y., Otten, L. A., Philpott, D., Reed, J. C., Reith, W., Schreiber, S., Steimle, V., and Ward, P. A. (2008) *Immunity* **28,** 285–287
6. Ting, J. P., Willingham, S. B., and Bergstralh, D. T. (2008) *Nat. Rev. Immunol.* **8,** 372–379
7. Kanneganti, T. D., Lamkanfi, M., and Núñez, G. (2007) *Immunity* **27,** 549–559
8. Sutterwala, F. S., Ogura, Y., Zamboni, D. S., Roy, C. R., and Flavell, R. A. (2006) *J. Endotoxin. Res.* **12,** 251–256
9. Lamkanfi, M., and Dixit, V. M. (2009) *Immunol. Rev.* **227,** 95–105
10. Bryant, C., and Fitzgerald, K. A. (2009) *Trends Cell Biol.* **19,** 455–464
11. Martinon, F., Mayor, A., and Tschopp, J. (2009) *Annu. Rev. Immunol.* **27,** 229–265
12. Hoffman, H. M., Rosengren, S., Boyle, D. L., Cho, J. Y., Nayar, J., Mueller, J. L., Anderson, J. P., Wanderer, A. A., and Firestein, G. S. (2004) *Lancet* **364,** 1779–1785
13. Martinon, F., Burns, K., and Tschopp, J. (2002) *Mol. Cell* **10,** 417–426
14. Kanneganti, T. D., Ozören, N., Body-Malapel, M., Amer, A., Park, J. H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E. P., Akira, S., and Núñez, G. (2006) *Nature* **440,** 233–236
15. Thomas, P. G., Dash, P., Aldridge, J. R., Jr., Ellebedy, A. H., Reynolds, C., Funk, A. J., Martin, W. J., Lamkanfi, M., Webby, R. J., Boyd, K. L., Doherty, P. C., and Kanneganti, T. D. (2009) *Immunity* **30,** 566–575
16. Miller, E. J. (1971) *Biochemistry* **10,** 1652–1659
17. Brand, D. D., Latham, K. A., and Rosloniec, E. F. (2007) *Nat. Protoc.* **2,** 1269–1275
18. Lamkanfi, M., Malireddi, R. K., and Kanneganti, T. D. (2009) *J. Biol. Chem.* **284,** 20574–20581
19. Holmdahl, R., Malmström, V., and Vuorio, E. (1993) *Ann. Med.* **25,** 251–264
20. Wannamaker, W., Davies, R., Namchuk, M., Pollard, J., Ford, P., Ku, G., Decker, C., Charifson, P., Weber, P., Germann, U. A., Kuida, K., and Randle, J. C. (2007) *J. Pharmacol. Exp. Ther.* **321,** 509–516
21. Kolly, L., Karababa, M., Joosten, L. A., Narayan, S., Salvi, R., Pétrilli, V., Tschopp, J., van den Berg, W. B., So, A. K., and Busso, N. (2009) *J. Immunol.* **183,** 4003–4012
22. Maelfait, J., Vercammen, E., Janssens, S., Schotte, P., Haegman, M., Magez, S., and Beyaert, R. (2008) *J. Exp. Med.* **205,** 1967–1973
23. Coeshott, C., Ohnemus, C., Pilyavskaya, A., Ross, S., Wieczorek, M., Kroona, H., Leimer, A. H., and Cheronis, J. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 6261–6266
24. Irmler, M., Hertig, S., MacDonald, H. R., Sadoul, R., Becherer, J. D., Proudfoot, A., Solari, R., and Tschopp, J. (1995) *J. Exp. Med.* **181,** 1917–1922
25. Adkison, A. M., Raptis, S. Z., Kelley, D. G., and Pham, C. T. (2002) *J. Clin. Invest.* **109,** 363–371